Environmental DNA Analysis – Non-invasive Detection of Endangered Species in Aquatic and Terrestrial Environments

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In wildlife investigations of endangered species – whether in wildlife forensics or in a conservation context - the determination or confirmation of species identity is essential and usually accomplished by DNA analysis. In the conservation context it is imperative that such testing is not resulting in further disturbance of the already endangered species, thus requiring the least invasive mode of sampling possible. The most non-invasive testing or confirmation of the presence of a species in a given environment can be accomplished by detection of the DNA they leave behind, be it by release of DNA directly into a soil or aquatic environment (e.g. Thomsen et al 2012), or by leaving behind DNA-containing material such as hair or scat (e.g. Hansen & Jacobsen 1999). In either case, DNA recovered will be trace amounts of degraded DNA and consequently requires highly sensitive methodology for detection and analysis, respectively an analysis approach which is optimized for the detection of highly degraded DNA.

Confirmation of the presence of aquatic species by detection of environmental DNA in water samples
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The presence of a species in an aquatic environment – like a pond, lake or stream – can be confirmed by detecting the DNA members of the species leave behind in the environment, by analysing DNA extracted directly from samples of the body of water (Ficetola et al. 2008, Jerde et al. 2011). Faeces, urine and epidermal cells are believed to be the predominant sources of environmental DNA (Lydolph et al. 2005; Haile et al. 2009), which may survive for up to two weeks after the species left the aquatic environment (Thomsen et al 2012). This trace DNA can be extracted from the water and detected utilizing species-specific primers in highly sensitive real time PCR assays. This was demonstrated for aquatic species like fish, amphibia like newts and as well as semi-aquatic mammals like the otter and insects like the dragon fly among others (Thomsen et al 2012).

In our current project, we build on experience gained in validation and utilization of existing methodology (Halford et al 2016) and go beyond the previously published by developing new assays for endangered species which so far have not been detected using this approach. Our first new assay was developed for the detection of the British water vole (Arvicola amphibius), the population of which has been in severe decline for decades (Strachan & Jeffries 1993, Strachan et al. 2000, Batsaikhan et al 2016).
Species-specific primers were designed based on a consensus sequence generated from 50 haplotype reference sequences of the A. amphibius Cytochrome b gene, obtained from Genbank. This consensus sequence was aligned with the Cyt b sequences of 68 related and/or sympatric species (ClustalW: Thompson et al. 1994) to identify the most species-specific sites for manual primer design. Primer-Blast (Ye et al. 2012) was utilised to confirm species-specificity of the forward primers and to design specific reverse primers, followed by OligoAnalyzer 3.1 (Owczarzy et al. 2008) to test for secondary structures.
Three primer pairs were selected for validation in standard PCR reactions, testing viability and species-specificity of the selected primer pairs, as well as general specificity and efficiency of the reaction at different annealing temperatures. Selecting the most efficient primer pair tested, a TaqMan probe and real-time PCR assay was designed, utilizing the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems). The essay was validated for species specificity against non-target species and sensitivity for target-species DNA (1.2µg/ml to 0.012pg/µl), with a current detection limit of 1.2pg/µl water vole DNA. This will be followed by further optimization to increase sensitivity of the assay.

Non-invasive detection of terrestrial species by analysis of species-specific DNA in scat
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The second project focuses on terrestrial species and their detection by utilizing DNA traces that can be found in their droppings (scat):
Studies in the wildlife conservation context traditionally utilize observational survey techniques, which can prove difficult to accomplish in case of species which are rare, nocturnal and/or elusive (e.g. Wilson & Delahay 2001, Kilshaw & Macdonald 2011). Consequently, a variety of approaches of DNA analysis-based methodology have been utilized to confirm the presence of the species in the environment for a number of years, by using non-invasively collected samples such as hair and scat (faeces). Scat samples contain traces of species-specific DNA due to accumulation of exfoliated epithelial cells from the intestinal wall of the defecating individual (Albaugh et al. 1992). DNA extracted from the sample material is analyzed by direct sequencing of mtDNA (e.g. Murakami 2002) or using SNP-based assays of mitochondrial (e.g. McEwing et al 2011) and nuclear DNA (Nussberger et al 2013). The critical requirement for any kind of assay based on non-invasive samples like scat is that it is sensitive enough to detect trace quantities of beyond that degraded DNA, as commonly recovered from this kind of material (Taberlet et al. 1999).

The current project project focuses on the wild cat (Felis silvestris), the British subspecies of which – the Scottish wild cat (Felis silvestris grampia) – is severely endangered (Yamaguchi et al 2015). The goal here is the development of a species-specific assay, which is sensitive enough to allow for detection of wild cat (Felis silvestris) DNA from scat samples without the need for real-time PCR based detection, by designing species-specific primers targeting short amplicons located in genes like cytochrome b or cytochrome oxidase I and utilizing optimized methodology to neutralize enzyme inhibitory substances commonly present in this kind of sample material. To account for the high probability of hybridization with house cats (Felis cattus), parallel utilization of autosomal nuclear loci has to be considered.

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